UV Spectroscopic Characterization of Type I Collagen

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Abstract

The near UV absorption of type I collagen can be used to measure its tyrosine content and, therefore, the integrity of the nonhelical telopeptides. The far UV absorption of the protein can be used to determine its concentration and to monitor its thermal denaturation. The concentration, when determined along with low speed centrifugation to precipitate collagen fibrils, allows monitoring of the *in vitro* assembly of collagen fibrils. These methods are examined in detail and compared with other available techniques in terms of the advantages, drawbacks, and pitfalls in their usages.

Key words: physicochemical properties, type I collagen, UV spectroscopy.

Introduction

Type I collagen has a low content of aromatic amino acids and a high content of glycine, proline, and hydroxyproline. Its secondary structure is unique in that three polypeptides, each of them being a left-handed helix, intertwine into a right-handed triple helix. Within the triple helix, the primary structure of the polypeptide chains consists of repeats of the triplet Gly-X-Y where X and Y can be any amino acid other than tyrosine and tryptophan but are often proline and hydroxyproline. Each of the helical polypeptides is flanked at its amino and carboxyl terminals by two short telopeptides which assume globular structures. In solution, the type I collagen molecule behaves like a semi-rigid rod with a length of 300 nm and a diameter of 1.5 nm. In extracellular matrices, type I collagen self-associates and exists in a fibrillar form. (For reviews of collagen structure and chemistry, see Piez, 1984 and Miller, 1984.)

In the past three decades, research aimed at understanding the structure-function relationship of type I collagen has been focused on examining the triple helical structure of the protein, the geometric arrangement of collagen within the fibrils, and the mechanism through which monomeric collagen self-assembles into fibrils. In essentially all studies of collagen in solution, for proper interpretation of results, it is necessary to know the concentration of the protein and be able to show that it exists in the native triple helical state. In studying the *in vitro* assembly of solubilized collagen into fibrils, it is also important to know the state of the nonhelical telopeptides of the protein. These telopeptides, digestible by endogenous proteases during extraction, play crucial but not yet fully understood roles in the fibril assembly (Helseth et al., 1979; Helseth and Veis, 1981; Capaldi and Chapman, 1982; 1984). Since all tyrosine residues of type I collagen are

found at the nonhelical telopeptides, their number can be used to gauge the intactness of the protein. Although methods are available to determine the concentration of collagen and its tyrosine content and to monitor the thermal denaturation and *in vitro* fibril assembly of the protein, they usually suffer from one or more drawbacks, i.e., poor accuracy, are time consuming, and/or require sophisticated and expensive instrument. In view of these needs, we investigated new methods of characterizing the physicochemical properties of type I collagen using UV absorption spectroscopy. The results of this study and a discussion of the proper applications of the spectroscopic methods are reported here.

Materials and Methods

Type I collagen was solubilized from calf skin with 0.5 M HOAc and purified as previously described (Na et al., 1986a). Further treatment of collagen with pepsin to remove its nonhelical telopeptides was accomplished following the method of Gelman et al. (1979). Pepsin (3200–3800 unit/mg) was from Sigma Co[1]. UV-grade guanidine hydrochloride (GuHCl)[2] was purchased from U.S. Biochemical Corp. and its solutions were filtered through sintered glass funnel before use.

Measurement of Collagen Concentration

The primary concentration of the stock collagen solution was determined by precision dry weight measurement following the protocol of Kupke and Dorrier (1978). Since collagen has very low absorption in the near UV region, secondary methods of concentration determination were developed by diluting the stock collagen solution with either AS (0.01 M NaOAc, 0.02 M NaCl, pH 4.0) buffer or Gu-HCl solution and measuring its far UV absorption with a Perkin-Elmer Lambda-7 UV-visible spectrophotometer. When diluting a sample in Gu-HCl solution, the final concentration of Gu-HCl was kept at 6 M by using a combination of 6 M and 8 M solutions.

Measurement of Thermal Denaturation

The thermal denaturation of collagen was monitored by measuring the hyperchromicity in the far UV region of the protein that accompanies the reaction. The results were compared with those obtained from circular dichroism (CD) spectroscopy and viscometry. A Perkin-Elmer Lambda-7 UV-visible spectrophotometer and a JASCO Model J41-C circular dichroism spectropolarimeter were used. Molar ellipticity at 222 nm was measured to follow the thermal denaturation of collagen. In the spectrophotometers, temperature of the collagen solution was regulated through use of jacketed cells connected to a Neslab Model RTE-8 heating/cooling water bath. In the absorption spectrophotometer, a jacketed cell holder was also connected in series to the water bath to improve the accuracy of the temperature. A linear increase of the sample temperature was achieved by using a Neslab Model ETP-3 electronic temperature programmer. With this setup, a small difference (0.05–0.1 °C) was still found between the actual temperature of the solution in the cuvette and the temperature of the circulating water. Consequently, the true temperature of the solution was measured by a digital thermometer with its microthermistor immersed in the sample solution above the light path.

The solution viscosity was measured with Cannon semimicro flow viscometers, the temperature was regulated with a Cannon Model M-1 constant temperature water bath. The samples were equilibrated for 30 min at each temperature. The reduced specific viscosities were measured at three different protein concentrations. They were then extrapolated to zero protein concentration to obtain the intrinsic viscosity.

Measurement of Aromatic Amino Acid Contents

The numbers of tyrosine and phenylalanine residues, the two aromatic amino acids found in type I collagen, were determined spectrophotometrically. Calf skin collagen, tyrosine, and phenylalanine were dissolved individually in 6 M Gu-HCl, 0.03 M NaP_i, pH 6.5 and their near UV absorption spectra were obtained with a Perkin-Elmer Lambda-7 UV-visible spectrophotometer, the latter was interfaced with a Telex-1260 microcomputer. To obtain the tyrosine and phenylalanine contents of the protein, the collagen spectrum in digital form was fitted through a least-squares procedure by theoretical spectra calculated from linear summations of the tyrosine and phenylalanine spectra.

The tyrosine and phenylalanine contents of collagen were also determined with a Beckman 119CL amino acid analyzer. The samples were hydrolyzed with 6 N HCl for 24 h before analysis.

Measurement of Fibril Assembly

The *in vitro* self-assembly of solubilized collagen into fibrils was carried out in PS buffer (0.03 M NaP_i, 0.1 M NaCl, pH 7.0). A small aliquot of the stock collagen, usually at approximately 5–10 mg/ml and dissolved in 1 mM HOAc, and an equal volume of a double strength PS buffer were added to 1 ml of PS buffer. Microman pipette (Rainin Co.) with a piston type disposable tip was used to measure and deliver the viscous collagen stock solution. The mixture was dispersed by gentle shaking at 4 °C, filtered through a 0.22 μM filter (Millipore), and then centrifuged at 36,000 × g for 30 min. To measure the fibril assembly reaction, the samples were added to 1.5 ml polypropylene microfuge tubes and incubated in a constant temperature water bath. After the incubation, the solutions were centrifuged with a DuPont Sorvall SH-MT rotor at 12,000 × g for 2 min. The protein concentrations of both the solution before the incubation and the supernatant after the centrifugation were determined spectroscopically as described above. The difference between these two values was taken as the amount of fibrils formed.

Results

Concentration

Since collagen has very low absorption in the near UV region, the feasibility of measuring its concentration from its far UV absorbance was investigated. Figure 1 shows far UV absorption spectra of calf skin collagen in AS buffer. An absorption peak was found with its apex located between 199 and 203 nm, shifting red with increasing collagen concentration. The inset of Fig. 1 shows the absorption at the apex as a function of the protein concentration. It was linear at low collagen concentrations where the

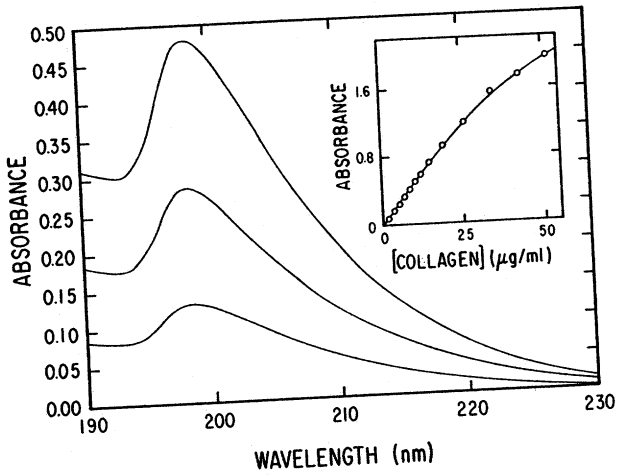

Fig. 1. *Far UV absorption spectra of calf skin collagen in AS buffer.* The collagen concentrations were 3.4, 6.9, and 10.3 μg/ml. The inset shows Beer's law plot of the absorption at the apex.

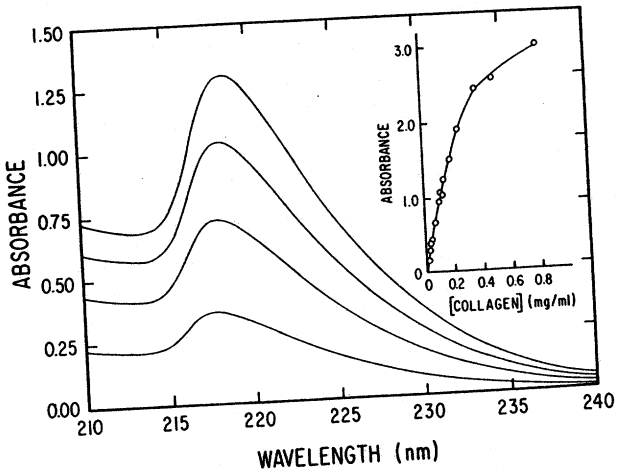

Fig. 2. *Far UV absorption spectra of calf skin collagen in 6 M Gu-HCl.* The collagen concentrations were 39, 77, 110, and 138 μg/ml. The inset shows Beer's law plot of the absorption at the apex around 219 nm.

optical density was below one, but became curvilinear at higher concentrations. In the linear region, an absorption coefficient of 49.0 cm^{-1}-ml-mg^{-1} was obtained.

Tests were also conducted to measure the concentration of collagen from its far UV absorption in 6 M Gu-HCl solution. Figure 2 depicts such far UV absorption spectra. In 6 M Gu-HCl, collagen displayed an absorption peak around 217–221 nm. The peak shifted red slightly at increasing protein concentration just like the absorption peak observed in AS buffer. In the inset of Fig. 2, the absorption at the apex of the peak was

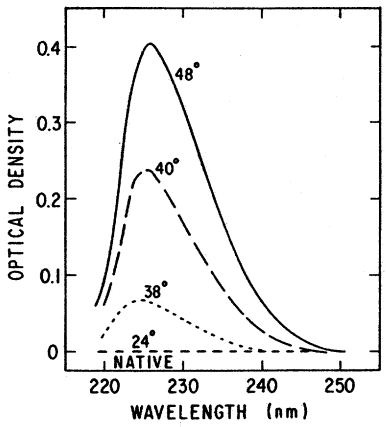

Fig. 3. *Difference absorption spectra of calf skin collagen.* Both the sample and reference contained 0.5 mg/ml collagen in AS buffer. The temperature of the sample is indicated next to each spectrum whereas the temperature of the reference cell was maintained at 20 °C.

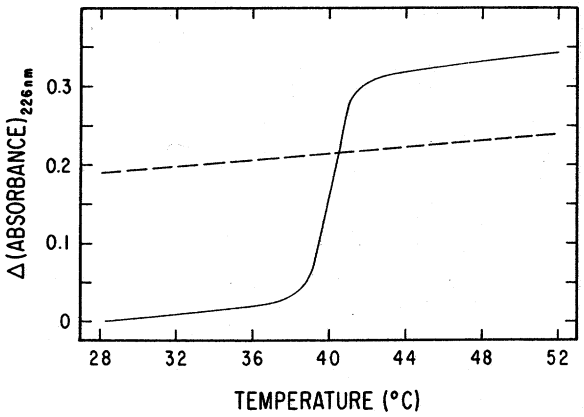

Fig. 4. *Measurement of thermal denaturation of calf skin collagen from its far UV hyperchromicity.* The solid curve depicts hyperchromicity of collagen at 226 nm. The sample was 0.5 mg/ml collagen in AS buffer. The reference was AS buffer. The dashed curve indicates change with temperature of absorption at 226 nm of the same concentration collagen dissolved in 6 M Gu-HCl.

plotted as a function of the protein concentration. The data were linear up to approximately 1.1 absorption unit with an absorption coefficient of 9.43 cm^{-1}-ml-mg^{-1}. Beyond this concentration range, the absorption coefficient decreased.

Thermal Denaturation

A hyperchromicity in the far UV absorbance of collagen accompanies the unfolding of the triple helical structure of the protein. Figure 3 depicts the temperature-dependent difference spectra of calf skin collagen solutions. The apex of the difference spectra was found at 226 ± 1 nm. The solid curve of Fig. 4 depicts the thermal transition of the collagen as monitored from the change in its absorption at 226 nm. A sharp sigmoidal transition was found at approximately 40 °C which superimposed on a small linear change of the solution absorbance throughout the entire temperature range examined. To investigate the cause of the latter absorption change, collagen was dissolved in a 6 M Gu-HCl solution and heated similarly. As shown by the dashed curve of Fig. 4, under this condition a linear increase of the solution absorbance also occurred with increasing solution temperature and the change was of the same magnitude as the linear change observed in the aqueous buffer. Furthermore, this linear absorption change was entirely reversible upon cooling. Since one expects the triple helical structure to unfold completely in 6 M Gu-HCl and that this unfolding reaction should only be partially reversible upon cooling (Privalov, 1982), the results of Fig. 4 suggested that the linear absorption change is unrelated to the unfolding of the triple helix. Consequently, in subsequent measurement of the thermal denaturation, a collagen solution of the same concentration but dissolved in 6 M Gu-HCl was used as the reference. Using this method, the apex of the difference spectrum was found at 223 nm. The melting profiles obtained by monitoring the hyperchromicity at 223 nm as shown in Fig. 5 are completely flat outside the transition zone.

The slow rate of unfolding of the collagen triple helix dictates a slow heating rate in measuring the melting temperature. Fig. 5 depicts the thermal denaturation of calf skin collagen in AS buffer measured by using the UV spectroscopic technique described above. Seven different heating rates ranging from 2 to 60 °C per hour were used. The inset of the figure shows the apparent melting temperature (T_m) as a function of the heating rate. The melting temperature measured at the two extreme heating rates differed by as much as 2 °C. The T_m's obtained at the heating rates of 1, 2, and 4 degrees per hour were linear with respect to the heating rate. Extrapolation to zero heating rate gave an equilibrium melting temperature ($T_m°$) of 38.5 °C for the calf skin collagen in AS buffer.

The possibility was also investigated that the UV light from the spectrophotometer could damage the primary structure of collagen and alter the thermal denaturation profile of the protein. The open circles of Fig. 6 shows the melting profile of a collagen sample that had been left inside the spectrophotometer, maintained at 4 °C, and irradiated at 223 nm for 15 h before heating. The denaturation of the same protein carried out without prior exposure to the UV light of the spectrophotometer is depicted by the open squares. It is evident that both denaturation curves are sharp and unimodal and that there is no measurable difference between them.

The melting profile of calf skin collagen obtained from the UV spectroscopic method was compared with those obtained from solution viscosity measurements and molar ellipticity determinations with a circular dichroism spectropolarimeter. The results shown in Fig. 7 indicate no significant differences between the transition temperatures obtained from these three methods. However, at the low temperature end, a slight reduction of the solution viscosity was noted prior to any detectable changes in the spectroscopic properties.

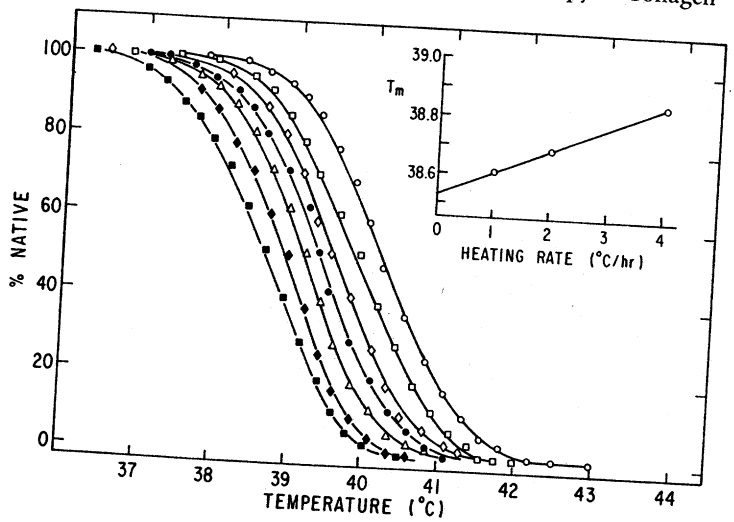

Fig. 5. *Thermal denaturation of calf skin collagen measured at different heating rates.* Calf skin collagen at 0.5 mg/ml was dissolved in AS buffer. The thermal transitions were obtained by measuring the UV hyperchromicity as described under Fig. 4 except the reference cell contained the same concentration collagen in 6 M Gu-HCl. The positions of the sample and reference cells were reversed so that the results reflected directly the percent collagen in the native state. The curves from left to right were obtained at heating rates of: 2, 4, 10, 15, 20, 40, and 60 deg/h. The inset shows the apparent melting temperature as a function of the heating rate.

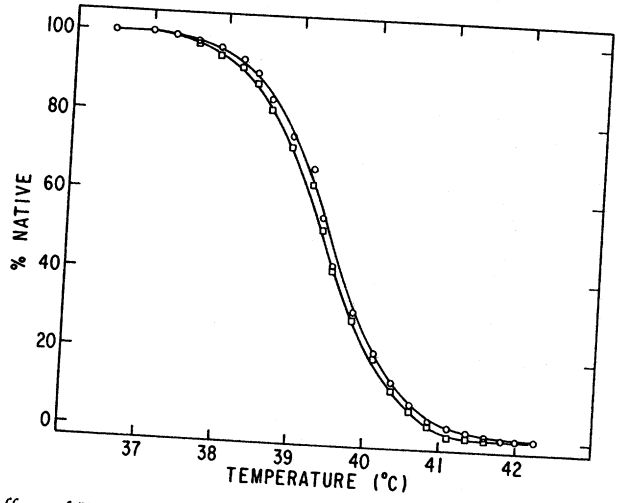

Fig. 6. *Lack of effect of UV light of spectrometer on the thermal denaturation of the calf skin collagen.* The circles were obtained as in Fig. 5 whereas the squares were obtained after the sample has been left in the cell chamber, maintained at 4 °C and irradiated by the light beam (223 nm) for 24 h.

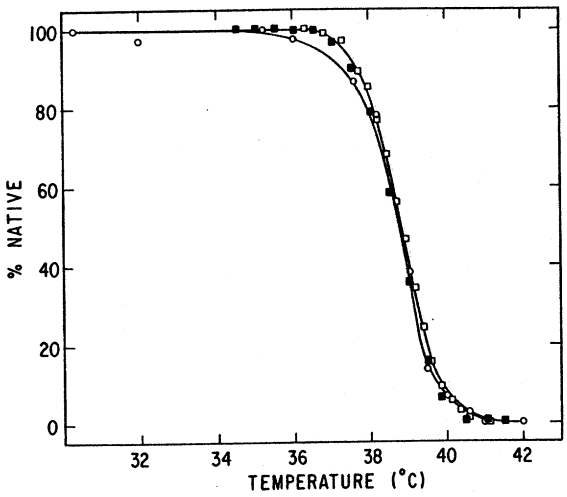

Fig. 7. *Comparison of collagen denaturations monitored by three different methods.* The thermal denaturation of collagen was monitored by UV hyperchromicity (□), circular dichroism (■), and viscosity (o). The heating rate was 30 min/deg in the spectroscopic methods. In viscosity measurements, the samples were incubated at a given temperature for 30 min before taking readings.

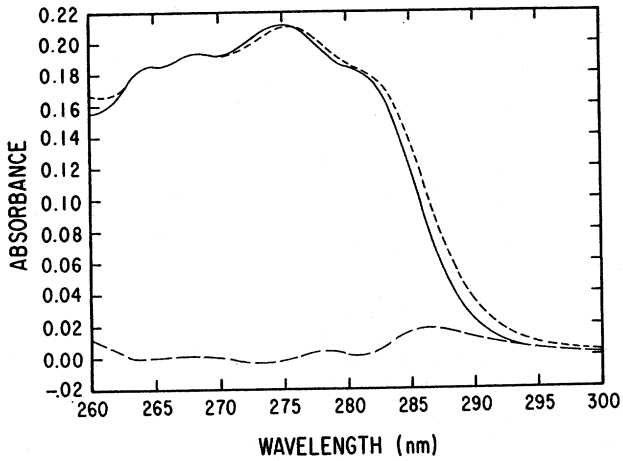

Fig. 8. *Fitting of a near UV absorption spectrum of calf skin collagen with theoretical spectra.* The experimental spectrum of collagen at 1.2×10^{-5} M (solid curve) was obtained in 6 M Gu-HCl, 0.03 M NaP_i, pH 6.5. Experimental spectra of tyrosine and phenylalanine (not shown) were obtained similarly. The best fitted theoretical spectrum (short-dashed curve) is a linear summation of the absorption of 1.42×10^{-4} M tyrosine and 5.06×10^{-4} M phenylalanine. The long-dashed curve is the difference spectrum between the two.

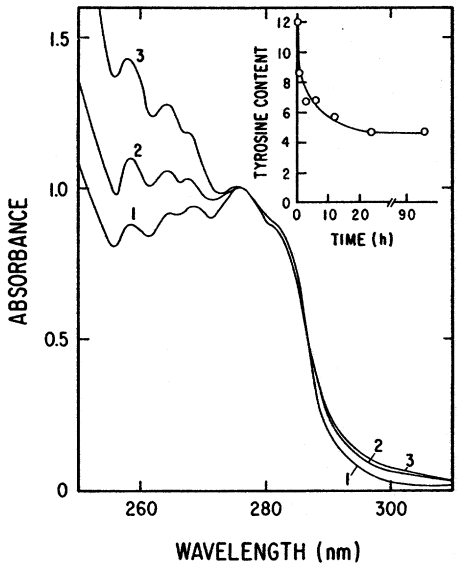

Fig. 9. *Near UV absorption spectra of native and pepsin digested calf skin collagens.* The spectra were normalized at 276 nm. Sample 1 was untreated collagen. Sample 2 and 3 were treated with 1 : 100 weight ratio of pepsin for 0.25 and 96 h, respectively, at 4 °C. The inset shows changes of the number of tyrosine residues versus the time of pepsin digestion.

Tyrosine Content

The solid curve of Fig. 8 depicts the near UV absorption spectrum of the collagen dissolved in a solution of 6 M Gu-HCl, 0.03 M NaP_i at pH 6.5. The spectrum exhibited a major absorption peak at 276 nm and a shoulder at 282 nm, reflecting the presence of tyrosine residues. Minor absorption shoulders were found at 268, 265, and 258 nm, indicating the presence of phenylalanine residues. Since type I collagen does not contain any tryptophan residues, no absorption peak or shoulder was observed at 290 nm and the absorbance at 276 nm can be attributed almost entirely to the tyrosine residues of the protein (Wetlaufer, 1962). Consequently, a molar absorption coefficient of 1500 l-mole^{-1}-cm^{-1} for tyrosine previously reported by Edelhoch (1967) can be used. The concentration of collagen was determined spectrophotometrically by further diluting the sample with 6 M Gu-HCl and measuring its absorbance at 218 nm. A collagen M_r of 285,000 used in the calculation was obtained from the amino acid composition of the protein (Miller, 1984). The result showed that the native collagen contained 12 ± 1 tyrosine residues per molecule.

To determine the number of phenylalanine residues per collagen molecule, the spectrum of Fig. 8 in the range of 250 to 310 nm was fitted through a reiterative procedure by theoretical spectra calculated from linear summations of the tyrosine and phenylalanine spectra. The short-dashed curve depicts the best fit theoretical curve and the long-dashed curve represents the difference between the experimental and theoretical spectra. The fitting gave 12 tyrosine and 42 phenylalanine residues per collagen molecule.

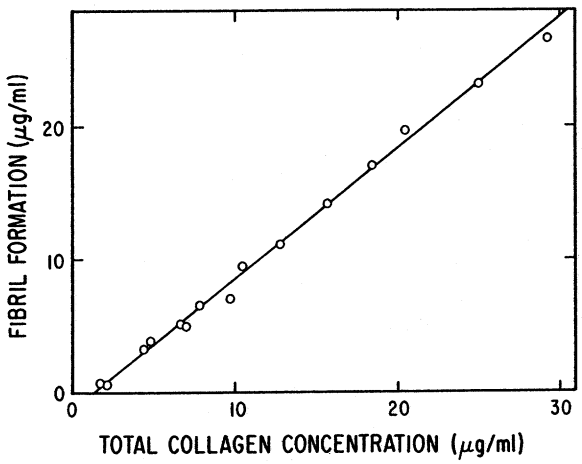

Fig. 10. *Equilibrium of collagen fibril assembly monitored by pelleting the fibrils and measuring the change in protein concentration.* Calf skin collagen was dissolved in PS buffer, incubated at 29.5 °C and centrifuged as described under Methods. The collagen concentration of the solution before the incubation and the supernatant after the centrifugation was determined by diluting in AS buffer and measuring the absorbance at the apex of the peak. The straight line is the linear least-squares fit of the data. It has a slope of 0.984 and intercepts x-axis at 1.05 ± 0.15 μg/ml.

The tyrosine and phenylalanine contents of the collagen were also determined with an amino acid analyzer. Assuming a collagen M_r of 285,000, the results showed 14.3 tyrosine residues and 42 phenylalanine residues per collagen molecule.

The three spectra in Fig. 9 depict a near UV absorption spectrum of untreated collagen together with those of two collagen samples that have been digested with pepsin for 0.25 and 96 h, respectively. It is evident that as the protein was digested, the intensity of the tyrosine peaks reduced relative to those of the phenylalanine ones. After extensive pepsin treatment, the absorption peak of phenylalanine at 258 nm emerged as the highest intensity peak replacing the one at 276 nm from tyrosine. The inset of Fig. 9 shows the change of tyrosine content as a function of enzyme digestion time. Under these conditions, the number of tyrosine residues dropped fast at the beginning of the incubation but leveled at approximately 4–5 tyrosine residues/collagen molecule after 24 h. Higher weight ratios of pepsin (up to 1 : 5) and longer digestion times were tested. However, the number of tyrosine residues did not decrease further and under no circumstances was the absorption of tyrosine at 276 nm and 285 nm completely eliminated, indicating that the telopeptides were never completely excised.

Fibril Assembly

Figure 10 shows collagen fibril assembly in PS buffer as a function of the total collagen concentration. The assembly reaction was allowed to reach equilibrium by incubating the solution at 29.5 °C for 24 h. The amount of fibrils assembled was then determined by pelleting the fibrils with centrifugation and measuring the decrease in collagen concentration from the far UV absorption in AS buffer. The solid line is the linear least-squares fitting of the data. A critical concentration of 1.05 ± 0.15 μg/ml was obtained from the intercept on the x-axis.

Discussion

A frequently encountered difficulty in collagen research is the lack of a simple, sensitive, yet accurate method of measuring the concentration of the protein. Due to the presence of few aromatic amino acids and the susceptibility to protease attack of the telopeptides where all of the tyrosine residues are located, it is not practical to determine the solution concentration of collagen from its near UV absorbance. Collagen shows a very low sensitivity in the Lowry colorimetric analysis (Lowry et al., 1951) and only a moderate sensitivity in an improved version of the Lowry test (Hartree, 1972). With the Bio-Rad assay (Bio-Rad Co.), another colorimetric method for determining protein concentration, the protein was observed to react with the reagent and precipitated in a fibrous form. Researchers in this field have resorted to the measurement of the molar ellipticity or the hydroxyproline content of the protein. The drawback of the former method lies in the requirement of a spectropolarimeter, a specialized and expensive instrument that is usually not available for routine use. Furthermore, the optical activity of the protein at 222 nm used in such measurements is derived from the triple helical structure of the protein. Consequently, the method requires that the protein remains in its native state to provide consistent results (Hayashi et al., 1979). The determination of hydroxyproline content is tedious (Jackson, 1967). It also suffers from low accuracy and sensitivity and is not suited for the routine analysis of large numbers of samples.

Our data shown in Figs. 1 and 2 indicate that the far UV absorption of collagen can be used in place of the near UV absorption to measure the protein concentration. In both AS and PS buffers, collagen showed strong absorption near 202 nm that is quite suitable for measuring low concentrations of native collagen. At these low concentrations the amount of light scattered by the protein is minimal and will not interfere with the measurement. One of our important applications of this method is in the study of the chemical equilibrium of the *in vitro* assembly of collagen. At conditions near physiological, it has been reported that essentially all collagen in the solution would assemble into fibrils if enough time were given for the reaction to reach its equilibrium, and it was suggested that the assembly reaction does not entail critical concentrations (Williams et al., 1978). It is possible, however, that there actually are critical concentrations for the assembly reaction but they are too low to be measured by the technique used. Indeed, by introducing glycerol which weakens the assembly reaction to the solution, we were able to observe critical concentrations for the assembly reaction as low as 36 μg/ml by measuring the far UV absorbance of the protein in 6 M Gu-HCl (Na et al., 1986a). As shown in Fig. 10, measuring the far UV absorption in AS buffer instead of 6 M Gu-HCl brought the sensitivity to the range of 0.5−10 μg/ml which permitted us to observe a critical concentration of 1.05 μg/ml for the fibril assembly at 29.5 °C in PS buffer without glycerol, indicative of a strong cooperative self-association.

Since the far UV absorbance of collagen in the pH 4.0 AS buffer increased upon denaturation of the protein, we also measured the absorbance of collagen in 6 M Gu-HCl solution where the protein was denatured and presumably in a random-coiled state. In 6 M Gu-HCl the absorption peak of collagen is shifted to a higher wavelength and is less likely to be interfered with by the absorbance of other solution components. Furthermore, the denatured collagen solution in 6 M Gu-HCl displayed essentially no turbidity and, therefore, is free from such complications. In the presence of 6 M Gu-HCl, collagen at concentrations up to approximately 0.1 mg/ml showed an absorption coefficient of 9.43 cm^{-1}-ml-mg^{-1} at 218 nm. Consequently, although the method can be used with denatured collagen, it is not as sensitive as measuring the protein absorbance

in AS buffer. The accuracy of the latter absorption coefficient is attested by the value of the partial specific volume of collagen obtained from solution density measurements where the concentration of the protein was derived from its far UV absorption in 6 M Gu-HCl (Na, 1986). Highly accurate collagen concentrations are essential for obtaining precise and accurate partial specific volumes of proteins from solution density measurements. We have obtained partial specific volumes of 0.685 ± 0.004 ml/g for the native collagen and 0.702 ± 0.002 ml/g for the denatured collagen, both in AS buffer (Na, 1986), which are consistent with previously published results (Boedtker and Doty, 1956; Rice et al., 1964). Another indirect indication of the accuracy of the extinction coefficient is found in the near UV determination of the aromatic amino acids of the protein. Agreements with published values were found in the tyrosine and phenylalanine contents of the protein determined from the near UV spectrum of the protein (Fig. 9). In this experiment, the collagen concentration was also derived from the far UV absorption of the sample in 6 M Gu-HCl.

Historically, the folding and unfolding of the collagen triple helix has been monitored by measuring either the optical rotation or viscosity of the solution (von Hippel and Wong, 1962; 1963). More recently, other molecular properties, such as circular dichroism (Hayashi et al., 1979), light scattering (Engel, 1962), and NMR (Chien and Wise, 1975) have been shown to exhibit similar transitions during the melting of collagen. Even with the diversity of the available methods, all suffer from one or more drawbacks of being time consuming, having poor accuracy, requiring specialized instruments, and being unsuitable for kinetic measurements. When using circular dichroism spectroscopy to monitor collagen denaturation, the UV light of the spectrometer was found to damage the structure and alter the thermal transition of the protein (Hayashi et al., 1979). In comparison, UV absorption spectroscopy provides rather simple and accurate measurement of collagen denaturation which is particularly suitable for kinetic studies. This method has been reported earlier (Wood, 1963; Danielsen, 1982, 1984, 1987). In the present study, it was demonstrated that the thermal transition of collagen monitored by far UV hyperchromicity is essentially identical to that obtained from CD. Minor differences did appear between the melting transition obtained from viscosity measurements and the spectroscopic ones (Fig. 7), the solution viscosity of collagen started to drop at a slightly lower temperature than the spectroscopic signals. Such a decrease in solution viscosity could be due to changes in intermolecular interactions rather than unfolding of the triple helical structure. A similar discrepancy between the melting profiles obtained from viscosity measurements and optical rotations has been reported by von Hippel and Wong (1963).

The results of Fig. 4 indicated that the small linear increase of solution absorbance with temperature is unrelated to the unfolding of the triple helix and can be eliminated by using a solution of the same protein in 6 M Gu-HCl as the reference. In addition, the denaturation profile was found not to be affected by the UV light of the spectrophotometer. The data shown in Fig. 5 also reiterated the important point that since the apparent melting temperature of collagen is strongly dependent on the heating rate, one must measure the apparent melting temperatures at several different heating rates and extrapolate them to zero heating rate in order to obtain the true equilibrium melting temperature.

The critical roles of the telopeptides of collagen in the *in vitro* fibril assembly have been demonstrated in several recent research reports (Helseth et al., 1979; Gelman et al., 1979; Helseth and Veis, 1981; Capaldi and Chapman, 1982; 1984). Since the tyrosine residues of type I collagen are located exclusively at the telopeptides whose amino acid

Discussion

A frequently encountered difficulty in collagen research is the lack of a simple, sensitive, yet accurate method of measuring the concentration of the protein. Due to the presence of few aromatic amino acids and the susceptibility to protease attack of the telopeptides where all of the tyrosine residues are located, it is not practical to determine the solution concentration of collagen from its near UV absorbance. Collagen shows a very low sensitivity in the Lowry colorimetric analysis (Lowry et al., 1951) and only a moderate sensitivity in an improved version of the Lowry test (Hartree, 1972). With the Bio-Rad assay (Bio-Rad Co.), another colorimetric method for determining protein concentration, the protein was observed to react with the reagent and precipitated in a fibrous form. Researchers in this field have resorted to the measurement of the molar ellipticity or the hydroxyproline content of the protein. The drawback of the former method lies in the requirement of a spectropolarimeter, a specialized and expensive instrument that is usually not available for routine use. Furthermore, the optical activity of the protein at 222 nm used in such measurements is derived from the triple helical structure of the protein. Consequently, the method requires that the protein remains in its native state to provide consistent results (Hayashi et al., 1979). The determination of hydroxyproline content is tedious (Jackson, 1967). It also suffers from low accuracy and sensitivity and is not suited for the routine analysis of large numbers of samples.

Our data shown in Figs. 1 and 2 indicate that the far UV absorption of collagen can be used in place of the near UV absorption to measure the protein concentration. In both AS and PS buffers, collagen showed strong absorption near 202 nm that is quite suitable for measuring low concentrations of native collagen. At these low concentrations the amount of light scattered by the protein is minimal and will not interfere with the measurement. One of our important applications of this method is in the study of the chemical equilibrium of the *in vitro* assembly of collagen. At conditions near physiological, it has been reported that essentially all collagen in the solution would assemble into fibrils if enough time were given for the reaction to reach its equilibrium, and it was suggested that the assembly reaction does not entail critical concentrations (Williams et al., 1978). It is possible, however, that there actually are critical concentrations for the assembly reaction but they are too low to be measured by the technique used. Indeed, by introducing glycerol which weakens the assembly reaction to the solution, we were able to observe critical concentrations for the assembly reaction as low as 36 μg/ml by measuring the far UV absorbance of the protein in 6 M Gu-HCl (Na et al., 1986a). As shown in Fig. 10, measuring the far UV absorption in AS buffer instead of 6 M Gu-HCl brought the sensitivity to the range of 0.5–10 μg/ml which permitted us to observe a critical concentration of 1.05 μg/ml for the fibril assembly at 29.5 °C in PS buffer without glycerol, indicative of a strong cooperative self-association.

Since the far UV absorbance of collagen in the pH 4.0 AS buffer increased upon denaturation of the protein, we also measured the absorbance of collagen in 6 M Gu-HCl solution where the protein was denatured and presumably in a random-coiled state. In 6 M Gu-HCl the absorption peak of collagen is shifted to a higher wavelength and is less likely to be interfered with by the absorbance of other solution components. Furthermore, the denatured collagen solution in 6 M Gu-HCl displayed essentially no turbidity and, therefore, is free from such complications. In the presence of 6 M Gu-HCl, collagen at concentrations up to approximately 0.1 mg/ml showed an absorption coefficient of 9.43 cm^{-1}-ml-mg^{-1} at 218 nm. Consequently, although the method can be used with denatured collagen, it is not as sensitive as measuring the protein absorb-

in AS buffer. The accuracy of the latter absorption coefficient is attested by the value of the partial specific volume of collagen obtained from solution density measurements where the concentration of the protein was derived from its far UV absorption in 6 M Gu-HCl (Na, 1986). Highly accurate collagen concentrations are essential for obtaining precise and accurate partial specific volumes of proteins from solution density measurements. We have obtained partial specific volumes of 0.685 ± 0.004 ml/g for the native collagen and 0.702 ± 0.002 ml/g for the denatured collagen, both in AS buffer (Na, 1986), which are consistent with previously published results (Boedtker and Doty, 1956; Rice et al., 1964). Another indirect indication of the accuracy of the extinction coefficient is found in the near UV determination of the aromatic amino acids of the protein. Agreements with published values were found in the tyrosine and phenylalanine contents of the protein determined from the near UV spectrum of the protein (Fig. 9). In this experiment, the collagen concentration was also derived from the far UV absorption of the sample in 6 M Gu-HCl.

Historically, the folding and unfolding of the collagen triple helix has been monitored by measuring either the optical rotation or viscosity of the solution (von Hippel and Wong, 1962; 1963). More recently, other molecular properties, such as circular dichroism (Hayashi et al., 1979), light scattering (Engel, 1962), and NMR (Chien and Wise, 1975) have been shown to exhibit similar transitions during the melting of collagen. Even with the diversity of the available methods, all suffer from one or more drawbacks of being time consuming, having poor accuracy, requiring specialized instruments, and being unsuitable for kinetic measurements. When using circular dichroism spectroscopy to monitor collagen denaturation, the UV light of the spectrometer was found to damage the structure and alter the thermal transition of the protein (Hayashi et al., 1979). In comparison, UV absorption spectroscopy provides rather simple and accurate measurement of collagen denaturation which is particularly suitable for kinetic studies. This method has been reported earlier (Wood, 1963; Danielsen, 1982, 1984, 1987). In the present study, it was demonstrated that the thermal transition of collagen monitored by far UV hyperchromicity is essentially identical to that obtained from CD. Minor differences did appear between the melting transition obtained from viscosity measurements and the spectroscopic ones (Fig. 7), the solution viscosity of collagen started to drop at a slightly lower temperature than the spectroscopic signals. Such a decrease in solution viscosity could be due to changes in intermolecular interactions rather than unfolding of the triple helical structure. A similar discrepancy between the melting profiles obtained from viscosity measurements and optical rotations has been reported by von Hippel and Wong (1963).

The results of Fig. 4 indicated that the small linear increase of solution absorbance with temperature is unrelated to the unfolding of the triple helix and can be eliminated by using a solution of the same protein in 6 M Gu-HCl as the reference. In addition, the denaturation profile was found not to be affected by the UV light of the spectrophotometer. The data shown in Fig. 5 also reiterated the important point that since the apparent melting temperature of collagen is strongly dependent on the heating rate, one must measure the apparent melting temperatures at several different heating rates and extrapolate them to zero heating rate in order to obtain the true equilibrium melting temperature.

The critical roles of the telopeptides of collagen in the *in vitro* fibril assembly have been demonstrated in several recent research reports (Helseth et al., 1979; Gelman et al., 1979; Helseth and Veis, 1981; Capaldi and Chapman, 1982; 1984). Since the tyrosine residues of type I collagen are located exclusively at the telopeptides whose amino acid

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sequences are known, the number of tyrosine residues per collagen can be used to determine the integrity of the telopeptides (Chandrakasan et al., 1976). NMR has been used for this purpose through measurement of the signal of the ring protons. The UV absorption measurement described here provides a much easier approach. The results shown in Fig. 8 indicated that the near UV spectrum of type I collagen can be reasonably well accounted for by a linear summation of the tyrosine and phenylalanine spectra. Such a fitting gave 12 tyrosine and 42 phenylalanine residues per collagen molecule which agree well with the results derived from amino acid analyses and the published amino acid composition of the protein (Miller, 1984). In Fig. 8, the experimental spectrum of collagen between 270–290 nm shifted slightly to the red compared to the theoretical one. This resulted in a difference spectrum (long-dashed curve) with peaks located at approximately 276 and 286 nm. A similar difference spectrum has been observed by Edelhoch (1967) when fitting the near UV spectrum of ribonuclease with the theoretical spectra calculated from glycyl-L-tyrosylglycine and cystine. The difference spectrum could be due to the imperfection of tyrosine as a model for the tyrosyl residues in collagen. Wetlaufer (1962) reported that the near UV spectrum of tyrosine changes with solution pH and is therefore influenced by the ionic charges near the phenolic group. It is interesting to note that the difference spectrum shown in Fig. 8 resembles very much the difference spectrum of tyrosine between pH 6 and 0.5 reported by Wetlaufer (1962).

Our results showed that the digestion of native collagen with pepsin at a weight ratio of 1:100 removed only a portion of the telopeptide. The number of tyrosine residues remaining on the protein as derived from UV spectroscopy leveled at approximately 4–5 per collagen after 24 h of digestion. Further increase of digestion time or the enzyme to a weight ratio of 1:10 or 1:5 did not result in further removal of the tyrosine residues from the protein. This observation is different from that of Gelman et al. (1979) with rat tail tendon collagen where essentially all tyrosine residues were removed after a 48 h treatment with a 1:5 ratio of pepsin. Perhaps the different results reflect variations in post-translational modifications of the collagen in different tissues which could block the access by and binding of the enzyme to portions of the telopeptides. Interestingly, a trifunctional crosslink involving histidine-92 of the $\alpha 2$ chain was shown recently to be unique in type I collagen from bovine skin but not from other skeletal tissues (Mechanic et al., 1987).

As far as monitoring the *in vitro* fibril assembly of collagen is concerned, currently the most widely used method is measurement of the solution turbidity, usually at a convenient wavelength chosen between 300 to 400 nm. A theoretical treatment of using the solution turbidity (total light scattering) as a quantitative measurement of the weight concentration of macromolecules with the shape of long thin rod was first presented by Berne (1973). The method was shown experimentally to be valid with tubulin-microtubule system by Gaskin et al. (1973). Although technically simple, the turbidimetric method has its drawbacks and pitfalls. One operational difficulty frequently encountered stems from the non-isotropic nature of the collagen aggregate. Collagen fibrils formed *in vitro* tend to further aggregate into large size clusters. They float around in the solution giving the so called "snowing effect" (Camper and Veis, 1977). This effect, particularly pronounced with protease-treated collagen at low protein concentrations, and/or after the solution has been shaken to disperse an additive, makes it impossible to measure accurately the weight concentration of collagen fibrils.

The second problem of the turbidimetric method is its unreliability as a quantitative measure of the fibrils in the solution. Theoretically, the solution turbidity measured in

the visible range is proportional to the weight concentration of the aggregates only if they assume the shape of long thin rod. More specifically, the length of the polymer must be greater than its diameter by a factor of more than six but must not exceed the limit of the Raleigh-Gans-Debye approximation (Timasheff, 1966; Gaskin et al., 1974; Berne, 1974; Timasheff, 1981). The morphologies of collagen fibrils known from electron microscopy suggested that they do meet the former requirement. However, the satisfaction of the Raleigh-Gans-Debye approximation remains in doubt. If the length of the fibril exceeds the limit for this approximation, usually in the range of several microns, the solution turbidity may not be proportional to the weight concentration of the fibrils. An empirical study we carried out recently showed that only below an optical density of one did the solution turbidity increase linearly with the fibril concentration (Na et al., 1986b). Furthermore, the same concentration of fibrils formed from collagen samples containing different amounts of oligomers displayed substantially different turbidities, perhaps reflecting differences in their physical dimensions (Na et al., 1986b). A similar observation has also been reported for the pepsin-treated collagen (McPherson et al., 1986). Consequently, one should exercise caution in making quantitative interpretation of the solution turbidity, particularly when comparing fibrils formed under different conditions. A decreased turbidity may not always mean a decreased amount of fibrils in the solution. For examining the chemical equilibrium of fibril assembly such as that shown in Fig. 10, and for comparative quantitative studies such as in investigating the effects of fibronectin and proteoglycans on the collagen fibril assembly, the method of pelleting the fibrils coupled with concentration measurements appears to be more suitable.

Footnotes

[1] Reference of company or product name does not constitute its endorsement by the U.S. Department of Agriculture over others of similar nature.

[2] The abbreviations used: AS, 0.01 M NaOAc, 0.02 M NaCl, pH 4.0; PS, 0.03 M NaP_i, 0.1 M NaCl, pH 7.0; Gu-HCl, guanidine hydrochloride; CD, circular dichroism.

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